Tofacitinib inhibits CD4 T cell polarisation to Th1 during priming thereby leading to clinical impact in a model of experimental arthritis

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Abstract

Objective

Janus kinases (JAK) are key cell membrane orientated tyrosine kinases that regulate inflammatory responses by transducing signals received by cytokine receptors that directly influence the polarisation and function of Th cells. Tofacitinib is a pan-JAK inhibitor approved for the treatment of RA. In this study, we explored the effects of tofacitinib in the outcomes of CD4+ T cell-dendritic cell (DC) interactions and their impact in autoimmune arthritis.

Methods

The impact of tofacitinib in CD4+ T cell outcomes during priming or re-activation were analysed using antigen-specific in vitro and/or in vivo systems. A breach of self-tolerance model of arthritis was used to investigate the effects of tofacitinib in the outcomes of newly primed and antigen experienced CD4+ T cells.

Results

Tofacitinib inhibited Th1 polarisation during priming both in vitro and in vivo. In vitro, impaired T-bet expression and IFN-γ production persisted upon secondary antigen challenge. Tofacitinib treatment during re-activation in vitro did not impact differentiation of antigen experienced CD4+ T cell towards Th1 phenotype. Moreover, JAK inhibition limited adaptive immune responses mediated by recently activated T cells and subsequent breach of self-tolerance in experimental arthritis.

Conclusion

Our findings provide a novel mode of action for tofacitinib, demonstrating a potential therapeutic utility via homeostatic immune restoration in very early autoimmune arthritis.

Key words

tofacitinib, early rheumatoid arthritis, rheumatoid arthritis, T cells, autoimmunity

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Introduction

Janus kinases (JAK) are intracellular non-receptor tyrosine kinases bound to cytokine receptors that transduce signals from a range of cytokines via the JAK-STAT (signal transducer and activator of transcription) pathway. Tofacitinib is a pan-JAK inhibitor that has been shown to be effective in models of autoimmune diseases (1–4), which led to its clinical development for the treatment of RA. As challenges remain in treating non-responder RA patients (5, 6), there is renewed interest in further understanding mechanisms of actions of existing therapeutic compounds to guide their optimal strategic utilisation, especially to drive immunologic homeostasis and thereby long term drug-free remission.

Among its immunomodulatory mechanisms, tofacitinib impairs $CD4^+$ T differentiation and pro-inflammatory cytokine production (1, 7). For the most part, this has focused on treatment from the initial encounter with antigen. However, as the initial breach of self-tolerance occurs some time prior to clinical onset, the responding $CD4^+$ T cell compartment will no doubt be a mixture of antigen experienced $CD4^+$ T cells primed in the absence of drug as well as newly primed cells responding to release of arthritic antigen. Disease stage, *i.e.* early onset *versus* flare and established chronic disease will undoubtedly be a relevant factor in the composition of this T cell population. The specific impact of tofacitinib in relation to T cell activity in the different phases of RA pathogenesis (*i.e.* priming vs re-activation of $CD4^+$ T cells) remains to be elucidated. Employing an antigen-specific T cell system, we investigated the impact of tofacitinib on $CD4^+$ T cell-DC interactions during priming and re-activation, both *in vitro* and *in vivo*. In addition, we employed a murine model of RA to evaluate the effects of tofacitinib treatment on the breach of self-tolerance. In this study, we demonstrate that tofacitinib inhibited Th1 differentiation during T cell priming but not during re-activation. In addition, tofacitinib limited breach of self-tolerance and immune responses mediated by newly activated $CD4^+$ T cells in experimental arthritis. These results provide a description of $CD4^+$ T cell phenotype and function altered by tofacitinib in different stages of T cell activation induction and highlight tofacitinib as a potential therapy for very early stages of RA.

Materials and methods

Mice

C57BL/6J mice (6- to 12-week-old) were purchased from Envigo (UK). OT-II transgenic mice with all immune cells expressing CD45.1 were bred in-house (Central Research Facilities, University of Glasgow). All animals were specific pathogen free and maintained in accordance with local and home office regulations.

Tofacitinib

Tofacitinib was purchased from LC labs (Boston, MA, US). Tofacitinib (free base) was reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for *in vitro* use. Tofacitinib (citrate salt) was reconstituted in DMSO and administered in vehicle solution (0.5% methylcellulose/0.025% Tween20 (Sigma) in Dulbecco's PBS (DPBS)) by gavage.

Adoptive transfer of antigen specific T cells and in vivo challenge

Isolated $CD4^+$ T cells from OT-II mice were stained with 1 μM CFSE (Invitrogen) and $1x10^6$ OT-II cells transferred intravenously into C57BL/6J recipients. One day later, mice received 25 μg $OVA_{323-339}$ peptide (pOVA) (Sigma-Aldrich) and 8 μg LPS (Sigma-Aldrich) in PBS injected into the hind footpads. Mice were treated by gavage with DMSO or tofacitinib citrate (25 mg/kg) once on the day of cell transfer and then twice daily on the consecutive 2 days. Three days after challenge, mice received a single treatment dose, were euthanised and their popliteal lymph nodes (pLN) harvested for flow cytometry analysis.

Breach of self-tolerance model of arthritis

Arthritis was induced in C57BL/6J recipients as previously described (8). In brief, Th1 polarised OT-II cells were adoptively transferred into C57BL/6 re-

cipients; mice were then s.c. immunised via the scruff with 100 μg OVA/CFA; articular inflammation was induced ten days later via periarticular challenge with 100 μg heat-aggregated OVA (HAO). Mice were treated with tofacitinib (25 mg/kg) or 0.5% DMSO twice daily by oral gavage from one day prior to challenge until the cull day. The development of arthritis was monitored as previously described (9). Popliteal LNs were harvested seven days after challenge for flow cytometry analysis. Hind foot ankle joints were removed and fixed in 10% neutral-buffered formalin for conventional histopathological processing. Arthritis was scored blind based on inflammation and synovial hyperplasia on a scale from 0 to 3.

DC-T cell co-culture and stimulation

Bone marrow-derived DCs (BMDC) were generated as previously described (10). On day 6, BMDCs were stimulated with LPS (100 ng/mL) for 24h prior to pulsing with pOVA on day 7 (5 μg/mL) (OVA_{high}) or (0.1 μg/mL) (OVA_{low}). BMDCs were co-cultured with CFSE-labelled OT-II $CD4^+$ T (1:10 DC:T cell ratio) in the presence of 100 nM Tofacitinib or 0.001% DMSO. Cells and supernatants were harvested after 72h for flow cytometry and Luminex assay respectively. For re-challenge cultures, cells that were treated with tofacitinib or DMSO during the first challenge received fresh complete media supplemented with 1 ng/ml of IL-2 (Biolegend) and rested for 3 days. Cells were then CSFE labelled and incubated with fresh pOVA-pulsed BMDCs. Alternatively, rested cells initially co-cultured in the absence of drug were incubated with fresh pOVA-pulsed BMDCs in the presence of tofacitinib or DMSO. Cells were harvested 3 days after re-challenge for flow cytometric analysis.

Flow cytometry

Single-cell suspensions were incubated with Fc Block for 10 min before adding fluorochrome-conjugated antibodies, as previously described (8). Fluorophore-conjugated antibodies were purchased from eBioscience, BD Biosciences or BioLegend. Prior to intracellular cytokine staining, cells were stimulated

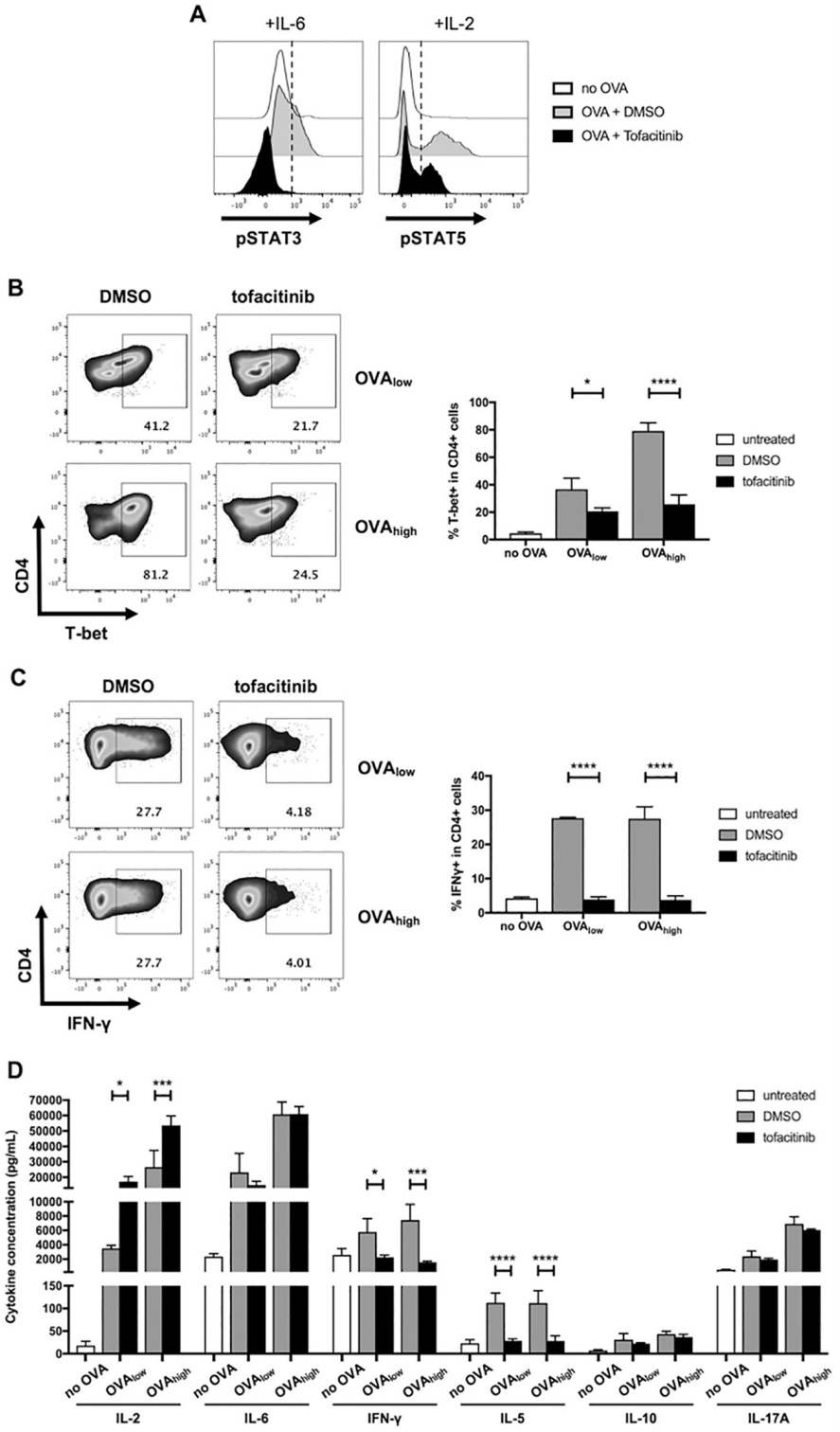

Fig. 1. Tofacitinib during priming *in vitro* impairs T-bet expression and IFN-γ production by $CD4^+$ T cells. OT-II $CD4^+$ T cells were cultured with BMDCs pulsed with suboptimal (OVA_{low}) or optimal (OVA_{high}) concentrations of pOVA in the presence of tofacitinib or DMSO.

A: Representative histograms of pSTAT3 or pSTAT5 expression in cells cultured for 24h and stimulated with IL-6 or IL-2, respectively.

B: T-bet or **(C)** IFN-γ expression in cells incubated for 72h.

D: Cytokine concentration measured from co-culture supernatants by Luminex.

*$p \leq 0.05$, ***≤ 0.001, ****≤ 0.0001.

BMDCs: bone marrow-derived dendritic cells; pOVA: ovalbumin peptide; DMSO: dimethyl sulfoxide; pSTAT: phosphorylated signal transducer and activator of transcription.

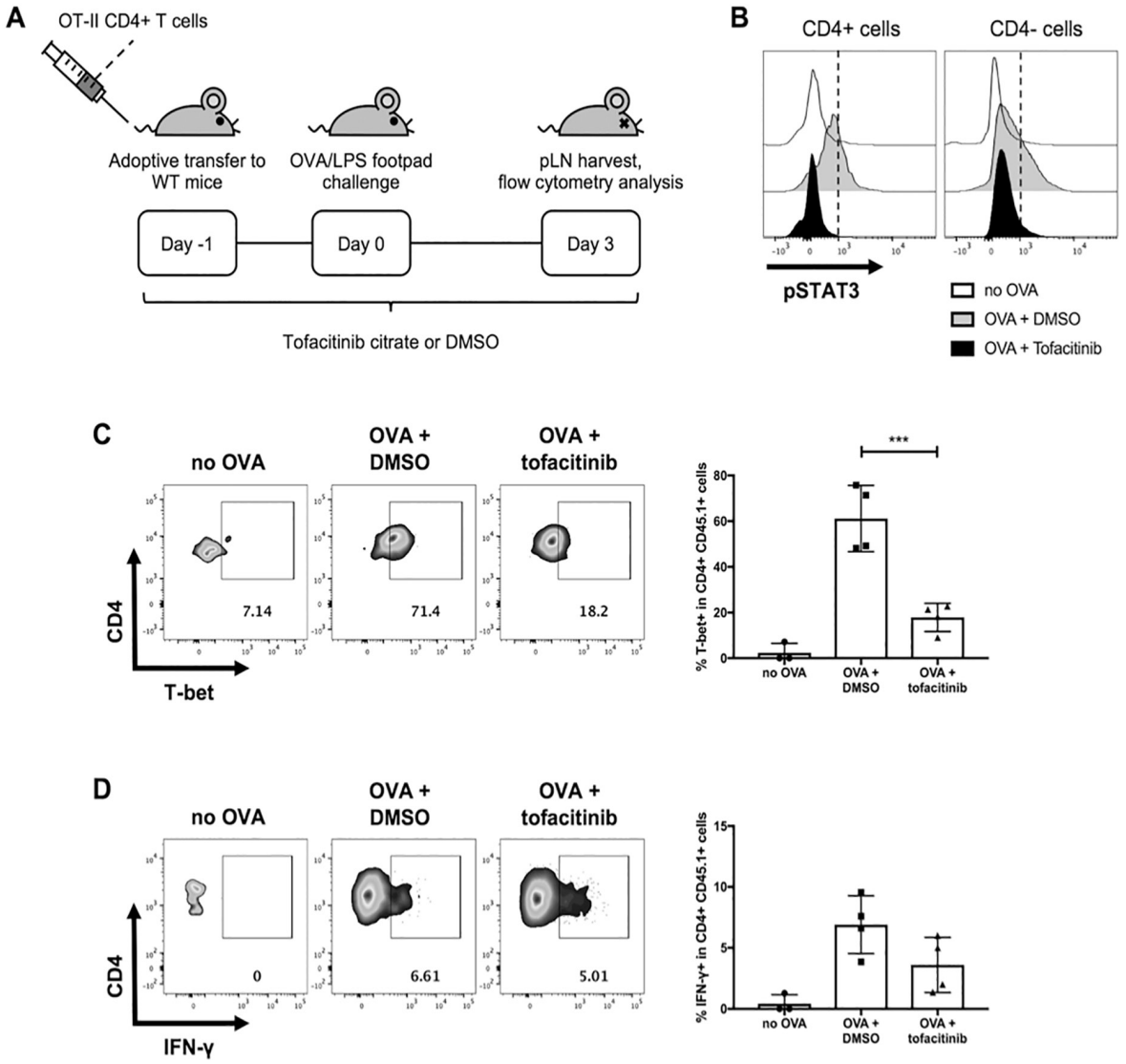

Fig. 2. Tofacitinib during priming *in vivo* suppresses T-bet expression in CD4+ T cells. C57BL/6J mice were submitted to the adoptive transfer model and treated with tofacitinib or DMSO (n=4).
A: Schematic representation of the adoptive transfer model. **B**: Representative histograms of pSTAT3 expression in PBMCs. **C**: Representative dot plots of T-bet expression and quantification of the proportion of T-bet+ cells in CD4+ CD45.1+ cells. **D**: Representative dot plots of IFN-γ and quantification of the proportion of IFN-γ+ cells in CD4+ CD45.1+ cells. ***$p \leq 0.001$.
DMSO: dimethyl sulfoxide; pSTAT: phosphorylated signal transducer and activator of transcription; PBMCs: peripheral blood mononuclear cells.

with phorbol myristate acetate (PMA) (Sigma-Aldrich) (20 ng/mL) and ionomycin (Sigma-Aldrich) (1 μg/mL) for 4 hours in the presence of brefeldin A. For intracellular cytokine and transcription factor staining, a Cytofix/Cytoperm (BD Biosciences) and a FoxP3 (eBioscience) kit was used, respectively, according to the manufacturers' instructions. Viability was assessed using a fixable cell viability dye (eBioscience) according to the manufacturer's instructions. Cells were acquired using a LSR Fortessa (BD Biosciences) or LSR-II (BD Biosciences) and analysed using FlowJo software (TreeStar).

Luminex

Cytokine release in co-culture supernatants was assessed using Milliplex Mouse cytokine panel (Merck). Concentrations of IL-2, IL-5, IL-6, IL-10, IL-17A and IFN-γ were determined using Bio-Rad Luminex 200 plate reader.

Statistics

Data are shown as mean ± SD. Statistical differences were determined using a one-way or a two-way ANOVA in experiments containing one or two categorical independent variables, respectively, using Prism version 6 (GraphPad Software, Inc, CA, USA).

Results

Tofacitinib impairs naïve CD4⁺ T cell differentiation into Th1 cells during priming

To examine the impact of tofacitinib on the priming of CD4⁺ T cells, we employed an antigen specific *in vitro* system, co-culturing OT-II T cells with pOVA-pulsed BMDCs. The activity of tofacitinib was confirmed in CD4 T cell priming co-cultures as reduced phosphorylation of STAT3 or STAT5 even in the presence of exogenous IL-6 or IL-2 respectively (Fig. 1A). Tofacitinib treatment during T cell priming *in vitro* did not impact the proportion

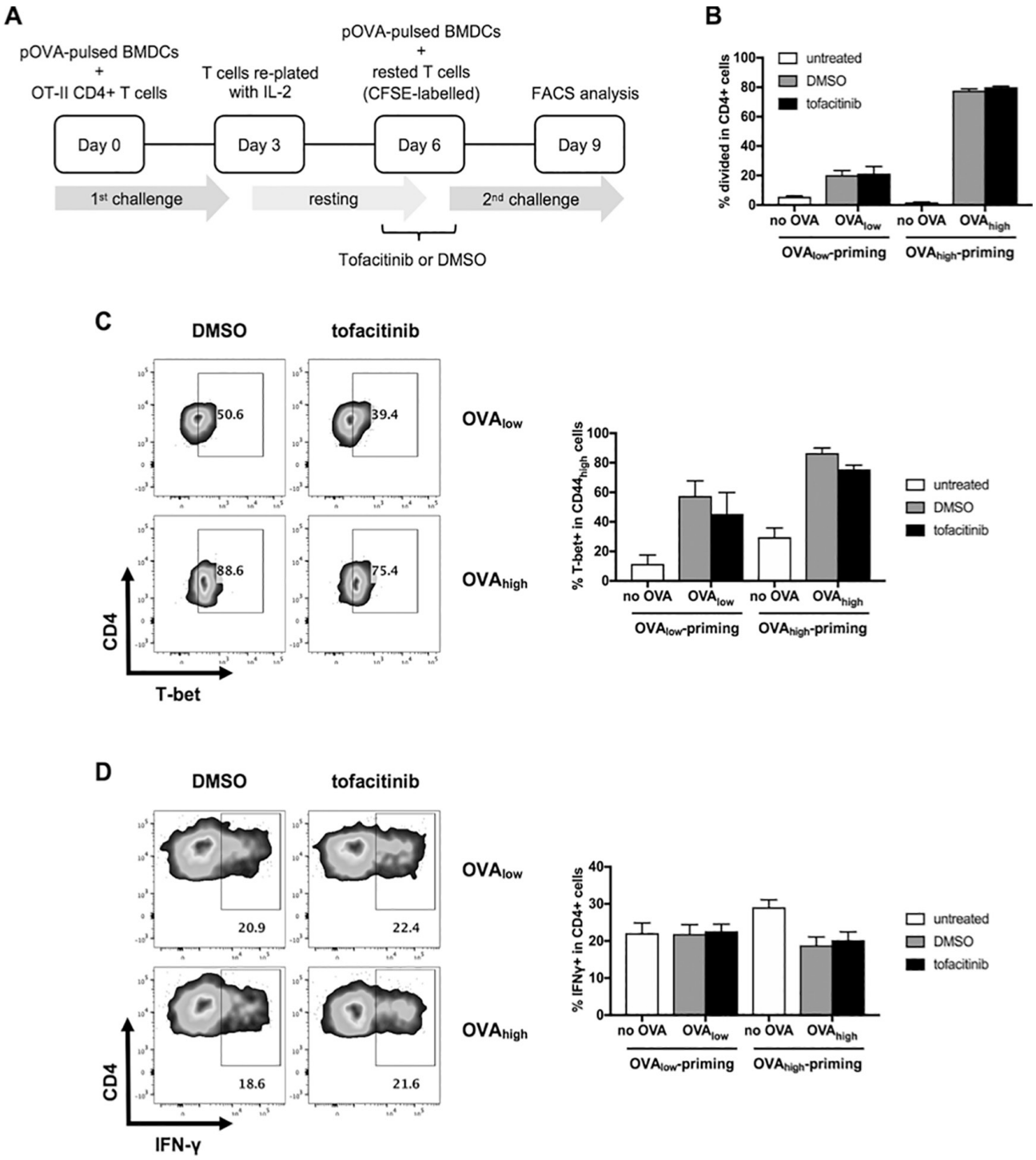

Fig. 3. Tofacitinib during re-activation does not impact T-bet expression or IFN-γ production by $CD4^+$ T cells.
A: OT-II $CD4^+$ T cells were cultured with BMDCs pulsed with pOVA for 72h, rested with IL-2 for further 72h and rechallenged with fresh pOVA-pulsed BMDCs for final 72h. **B**: Quantification of the proportion of cells that have divided in cells that were primed under suboptimal (OVA_{low}-priming) or optimal (OVA_{high}-priming) activation conditions and reactivated under similar conditions. **C**: T-bet expression in $CD44_{high}$ cells. **D**: IFN-γ expression in $CD4^+$ cells. *p*=non-significant for all comparisons.
BMDCs: bone marrow-derived dendritic cells; pOVA: ovalbumin peptide.

of cells with phenotypes indicative of recently primed ($CD44_{high}CD62L+$) or memory ($CD44_{high}CD62L-$) $CD4^+$ T cells but did consistently result in a minor reduction in proliferation (Supplementary Fig. S1). Given that the genetic background of OTII animals favours Th1 development (11) and considering that this model of autoimmune arthritis is mediated by Th1, but not Th2 cells (9), the expression of T-bet, the key Th1 transcription factor (12), was evaluated. The proportion of $CD4^+$ T cells expressing T-bet (Fig. 1B) was significantly reduced. Consistent

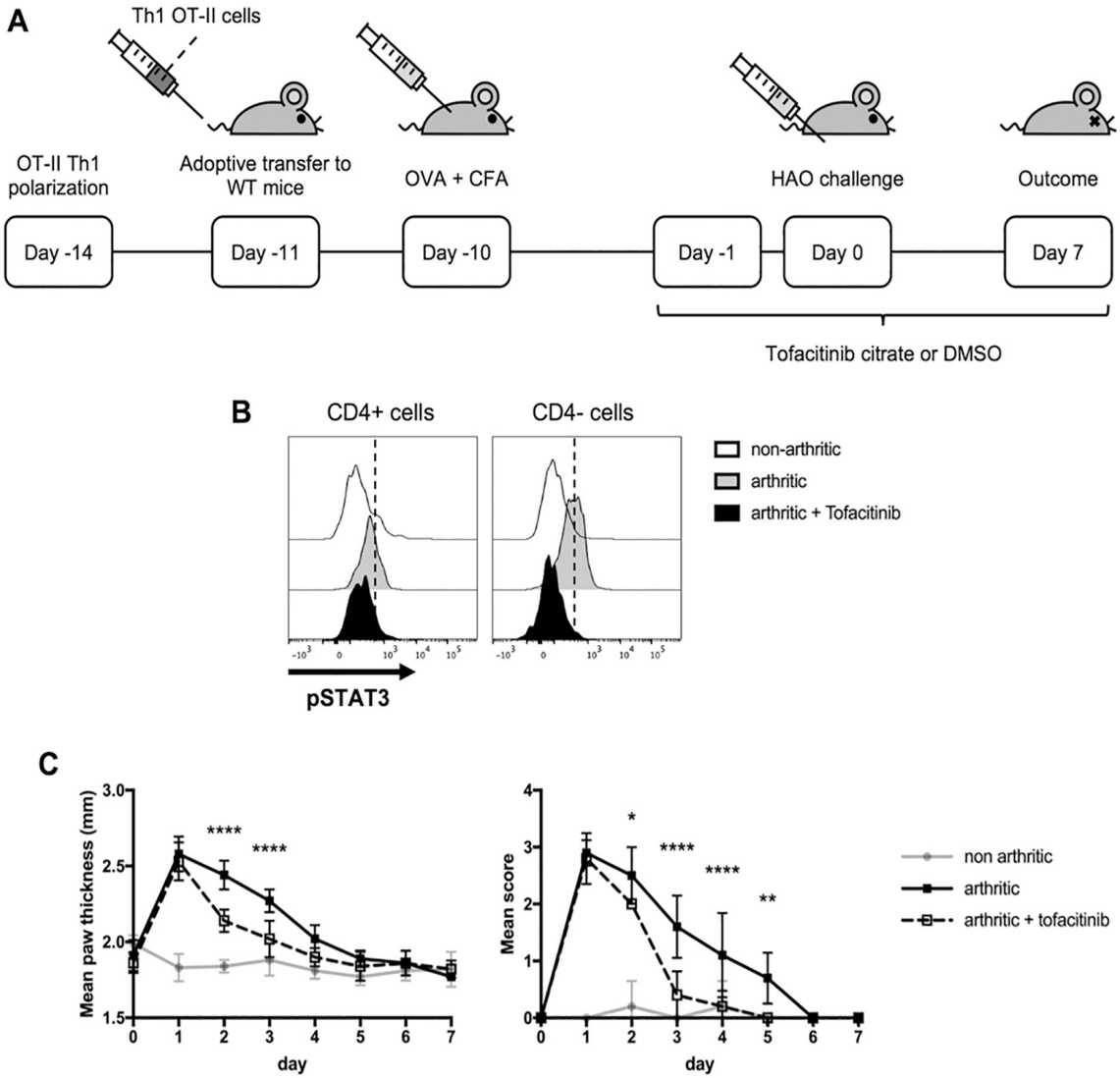

Fig. 4. Tofacitinib ameliorates clinical disease in experimental arthritis.
A: Arthritis was induced in C57BL/6J mice under treatment with tofacitinib or DMSO and the clinical course was monitored for 7 days after the footpad challenge (n=5). **B**: Representative histograms of pSTAT3 expression in PBMCs. **C**: Quantification of mean paw thickness and mean clinical scores.
*p≤0.05, **≤0.01, ****≤0.0001.
DMSO: dimethyl sulfoxide; pSTAT: phosphorylated signal transducer and activator of transcription; PBMCs: peripheral blood mononuclear cells.

with this finding, tofacitinib treatment reduced the capacity of these CD4 T cells to produce IFN-γ (Fig. 1C), a key cytokine mediating Th1 effector functions. A decrease in secretion of IL-5 was also observed in the presence of tofacitinib, while IL-17A, IL-10 and IL-6 production were unaffected (Fig. 1D). An increase in IL-2 secretion in the presence of tofacitinib was noted. Starting at 24 hours after initial TCR engagement, IL-2 produced by Th cells initiates autocrine STAT5-dependent negative feedback loop thereby limiting its own production (13). As such, notably increased IL-2 levels found in the supernatant would be consistent with an overcompensation arising from the loss of STAT5.

To establish whether tofacitinib would have a similar impact on CD4+ T cell priming *in vivo*, we employed an adoptive transfer model. CFSE-labelled CD45.1+ OT-II cells transferred into congenic recipients and subsequently challenged using OVA/LPS (Fig. 2A). *In vivo* treatment with tofacitinib decreased pSTAT3 expression in both CD4+ and CD4- PBMCs (Fig. 2B). No changes in T cell proliferation or activation in adoptively transferred T cells were found (data not shown). In accordance with our *in vitro* data, *in vivo* treatment with tofacitinib decreased the proportion of T-bet+ T cells in comparison with cells from control animals (Fig. 2C). However, differently from our *in vitro* data, no changes in the proportion of IFN-γ+ T cells (Fig. 2D) or in the median fluorescence intensity (MFI) of IFN-γ producer populations (data not shown) were found. *In vivo*, tofacitinib might be indirectly modulating T cell cytokine signalling pathways, partially restoring T cell IFN-γ production.

To analyse whether the effects of tofacitinib during priming persists upon subsequent antigen exposure, CD4+ T cells that were previously primed *in vitro* in the presence of tofacitinib and

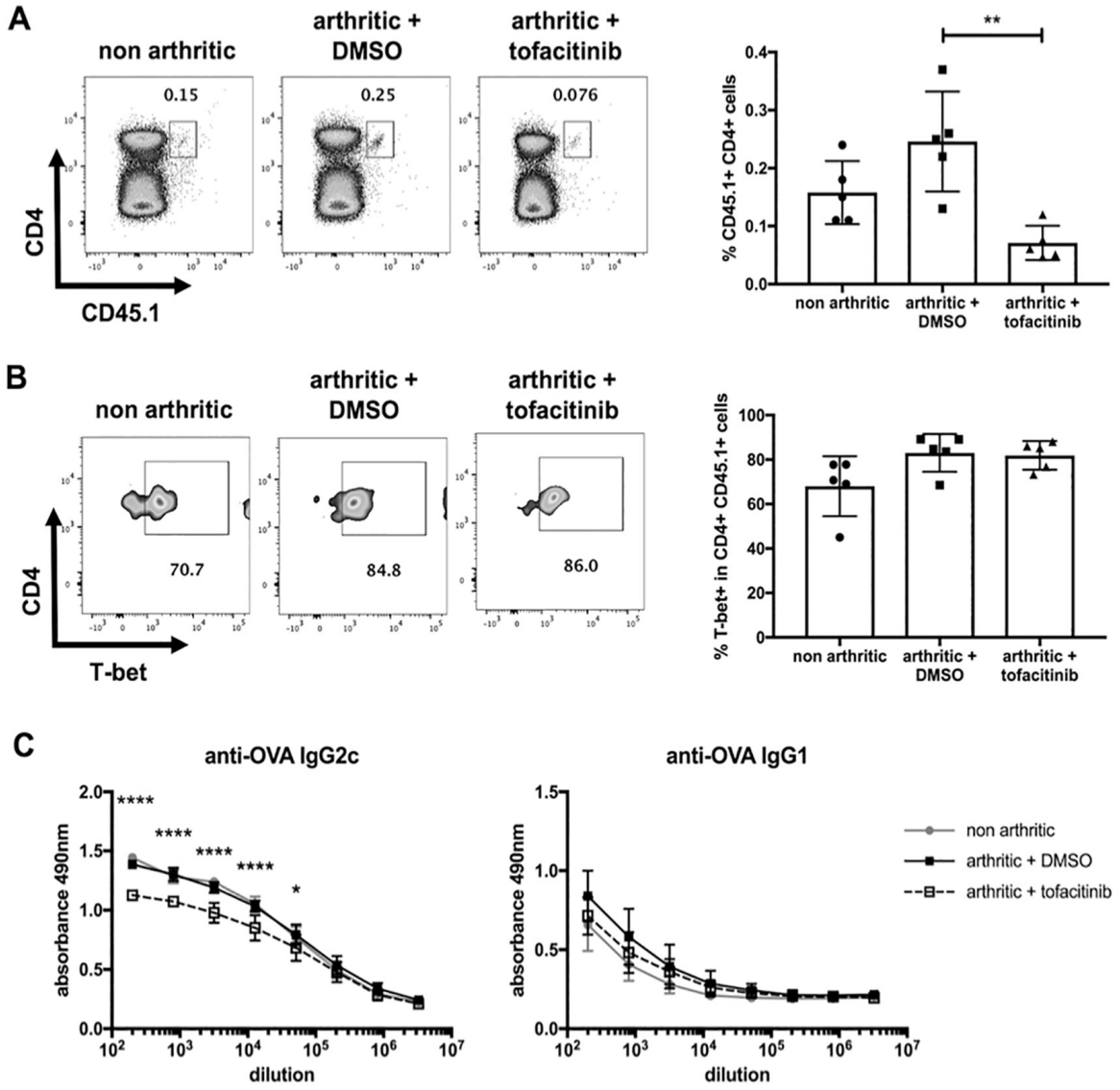

Fig. 5. Tofacitinib inhibits the accumulation of OT-II cells in the pLN and decreases anti-OVA IgG2c production. Popliteal LNs of mice under experimental arthritis were analysed by flow cytometry 7 days after the footpad challenge (n=5).
A: Gating strategy for the identification of adoptively transferred OT-II cells and quantification of the proportion of CD4+ CD45.1+ cells. **B**: Representative dot plots of T-bet expression and quantification of the proportion of T-bet+ cells in CD4+ CD45.1+ cells. **C**: Quantification of the absorbance of anti-OVA IgG2c or IgG1 antibodies from mice serum.
*$p \leq 0.05$, ** ≤ 0.01, **** ≤ 0.0001.

rested before rechallenge using antigen-pulsed BMDCs in the absence of drug (Suppl. Fig. S2A). In this assay, no differences were found in T cell proliferation (Suppl. Fig. S2B). However, CD4 T cells primed in the presence of tofacitinib continued to display a decreased propensity for expression of T-bet and IFN-γ upon rechallenge (Suppl. Fig. S2C-D). This suggests priming in the presence of tofacitinib has consequences for further antigen specific responses made following removal of the drug. The production of IL-2 was also analysed; however, no differences were detected between drug and DMSO groups (Suppl. Fig. S2E).

Tofacitinib does not impact Th1 attributes of antigen experienced CD4+ T cells upon re-activation

We next sought to determine whether tofacitinib would similarly impact responses of antigen experienced CD4+ T cells primed before exposure to the drug. CD4+ T cells were primed in the absence of tofacitinib before labeling with CFSE and co-culture with fresh antigen-pulsed BMDCs in the presence of drug (Fig. 3A). Tofacitinib did not affect T cell proliferation (Fig. 3B) or proportion of T-bet+ (Fig. 3C) or IFN-γ+ (Fig. 3D) expressing cells when originally primed in the absence of drug and then re-activated in the presence of tofacitinib. This data demonstrates that tofacitinib does not impact Th1 type responses made by antigen-experienced T cells that had been primed prior to drug treatment.

Tofacitinib limits breach of self-tolerance in experimental arthritis

To evaluate our observations of tofacitinib impact on different stages of

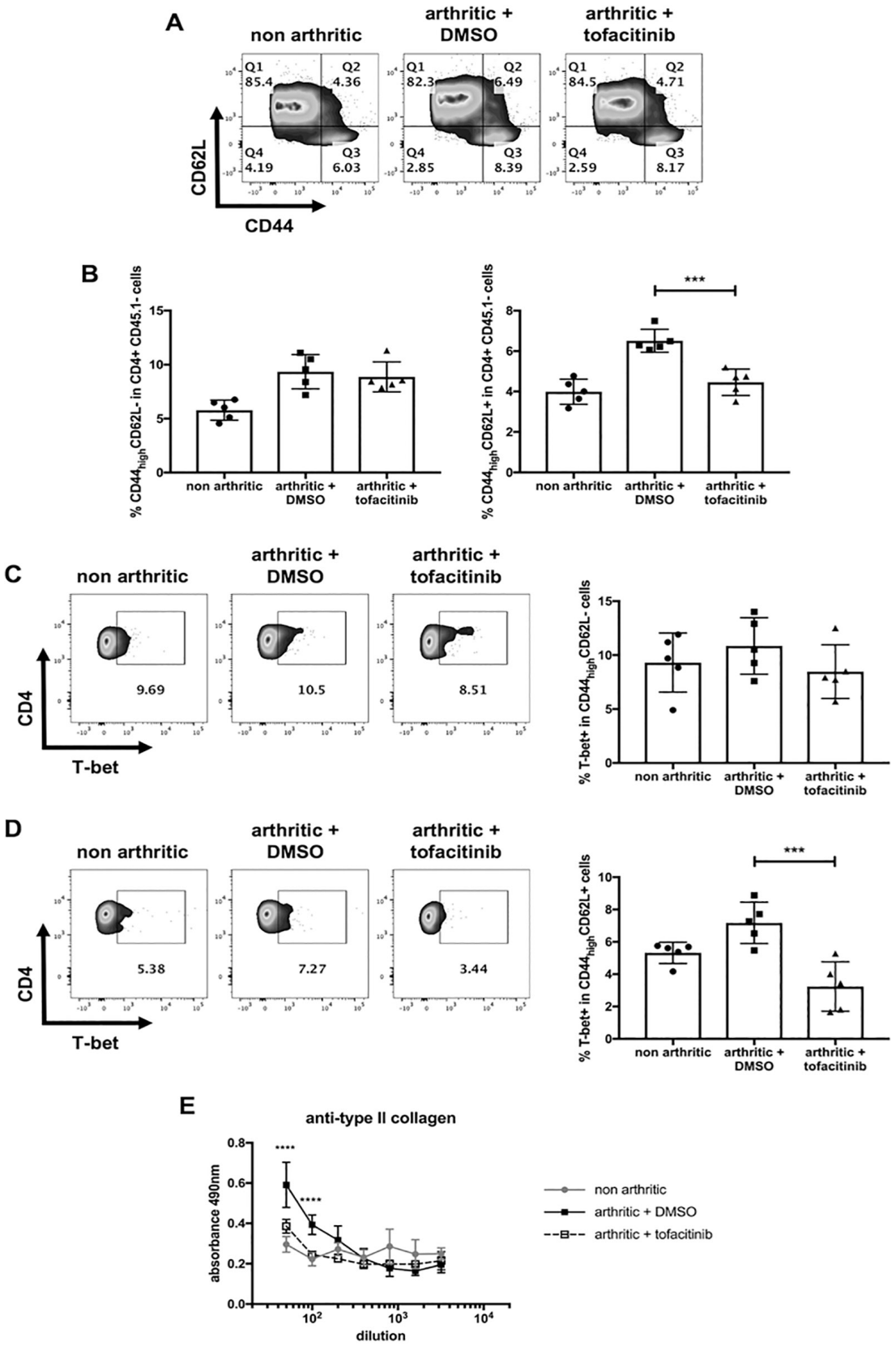

Fig. 6. Tofacitinib inhibits T-bet expression by $CD44_{high}CD62L+$ endogenous CD4+ cells and diminishes anti-type II collagen production. Popliteal LNs of mice under experimental arthritis were analysed by flow cytometry 7 days after footpad challenge (n=5). **A**: Gating strategy for the identification of phenotypes suggesting naïve ($CD44_{low}$ CD62L+), recently activated ($CD44_{high}$ CD62L+) or memory ($CD44_{high}$ CD62L-) T cells. **B**: Quantification of the proportion of $CD44_{high}$ CD62L- or $CD44_{high}$ CD62L+ cells in $CD4^+$ CD45.1- cells. **C**: Representative dot plots of T-bet expression and quantification of the proportion of T-bet+ cells in $CD44_{high}$ CD62L- or **(D)** $CD44_{high}$ CD62L+ cells. **E**: Quantification of the absorbance of anti-type II collagen antibodies from mice serum. ***$p \leq 0.001$, ****≤ 0.0001.

$CD4^+$ T cell responses we moved to a model of autoimmune arthritis. In this model, acute articular inflammation is driven by an adoptively transferred population of antigen specific Th1 cells in response to challenge with cognate antigen and gives rise to a breach of self-tolerance (Fig. 4A). This allowed us to examine responses made by both antigen-experienced (transferred Th1) and newly primed (endogenous cells) $CD4^+$ T cells during articular inflammation and establish the impact of tofacitinib in an arthritis context. Tofacitinib decreased the expression of pSTAT3 in $CD4^+$ or $CD4^-$ PBMCs, showing that the drug effectively blocked JAK/STAT pathways (Fig. 4B). Mice treated with tofacitinib showed decreased paw thickness in days 2 and 3 (Fig. 4C) and clinical scores in days 2, 3, 4 and 5, demonstrating a faster resolution of clinical disease following administration of the drug. In addition, reduced inflammatory cell infiltration was noted in joints from mice treated with tofacitinib although this was not found to reach significance (Suppl. Fig. S3).

To understand the effects of tofacitinib in the responses of T cells that have previously encountered antigen, in the context of arthritis, adoptively transferred cells, which play a key role in the breach of self-tolerance model, were analysed. Tofacitinib inhibited the accumulation of adoptively transferred cells in the LNs, demonstrated by a significant decrease in proportion of CD45.1+ cells (Fig. 5A). No differences were found in expression of T-bet+ in adoptively transferred cells (Fig. 5B), consistent with our *in vitro* data showing no impact of the drug on recall of antigen experienced $CD4^+$ T cells. Tofacitinib did however decrease the secretion of anti-OVA IgG2c antibodies, but not IgG1 antibodies (Fig. 5C). Although IgG2c subclass is commonly secreted during Th1-type immune responses (14), our data does not support an effect of the drug in the loss of Th1 activity. As such, tofacitinib could be directly affecting B cell responses, as previously described (15, 16).

To better understand the impact of tofacitinib in autoimmunity, endogenous LN $CD4^+$ cells, which were previously demonstrated to contain populations of auto-reactive T cells (9), were analysed. Although the drug did not affect the proportion of $CD44_{high}$-CD62L- cells, tofacitinib decreased the proportion of $CD44_{high}CD62L+$ cells (Fig. 6A, B), suggesting an impact in the activation of recently primed T cells. No differences were found in the proportion of T-bet+ cells in $CD44_{high}$-CD62L- cells (Fig. 6C). However, tofacitinib decreased the proportion of cells expressing T-bet in $CD44_{high}$-CD62L+ cells (Fig. 6D). In addition, we found decreased secretion of anti-type II collagen antibodies in the serum of tofacitinib-treated mice in comparison with DMSO-treated animals (Fig. 6E). These results, therefore, suggest that the impact of tofacitinib in clinical disease, in particular, the breach of self-tolerance, could be attributed to its inhibitory effect on recently primed $CD4^+$ T cell responses.

Discussion

Tofacitinib has proven efficacy in the treatment of RA, however, its mode of action *in vivo* remains to be fully elucidated. The drug's effects in the different stages of T cell activation and the participation of these components following tofacitinib treatment in RA remain unclear. We provide evidence that tofacitinib exposure during priming inhibits Th1 differentiation *in vitro* and *in vivo*. In addition, we have shown that this impaired T cell polarisation persists under a second antigen challenge. However, tofacitinib exposure during re-activation did not inhibit Th1 differentiation of antigen experienced $CD4^+$ T cells. The capacity of tofacitinib to modulate T cell functions following priming was shown to be relevant in the context of autoimmune arthritis. We demonstrated that tofacitinib limits breach of self-tolerance in experimental arthritis, evidenced by decreased production of autoantibodies, possibly influenced by an inhibition in Th1 development of recently activated cells.

Few studies investigating the impact of tofacitinib on human PBMCs reported diminished T cell activation (17, 18) that was subsequently restored upon drug withdraw (17). However, these reports were based in the use of CD25 as activation marker, a protein that functions as an IL-2 receptor α chain (IL-2Ra) on activated T cells. Tofacitinib disrupts the STAT5-dependent negative feedback loop that limits IL-2 production (13), increasing IL-2 secretion but preventing T cells from responding to IL-2 stimulation, which can, in turn, result in failure to upregulate CD25 expression (19). We found that *in vitro* or *in vivo* exposure of tofacitinib during T cell priming did not affect the expression of activation markers CD44/CD62L, suggesting that the drug's presence during priming does not affect T cell activation. In support, tofacitinib did not affect the expression of these activation markers on cells primed in the presence of tofacitinib and challenged a second time, as well as on cells that were primed in a drug-free environment and re-activated in the presence of the drug.

The analysis of tofacitinib impact during priming on $CD4^+$ T cell proliferation *in vitro*, but not *in vivo,* showed that T cells had lower levels of cell division at the drug concentration stablished to be required to inhibit JAK signalling (100 nM) (20), possibly due a diminished response to IL-2 stimulation. However, we demonstrate that this minor effect is lost upon a secondary antigen challenge and it is not evident in cells treated with tofacitinib during re-activation only. Tofacitinib exhibited no effect on naïve murine $CD4^+$ T cell proliferation when used at up to 1000 nM in a study conducted by Ghoreschi *et al.* (1). However, even at 100 nM dose the drug markedly reduced proliferation of lymphocytes derived from PBMCs of healthy donors and subjected to PHA stimulation *in vitro* (17). Interestingly, this effect was completely reversed upon drug removal. A comparable dose-dependent effect on cell division was observed in $CD4^+$ T cells purified from PBMCs and synovium samples from active RA patients and stimulated *in vitro* (anti-CD3/anti-CD28) in the presence of tofacitinib (21). In addition to these *in vitro* studies, Sonomoto and colleagues examined T cells from RA patients receiving tofacitinib treatment as a part of clinical trial (22). After 12 months of treatment, $CD4^+$ T

cell proliferative potential was notably suppressed and correlated with disease activity improvement. Altogether, these findings suggest that one of tofacitinib's mechanisms of action in its clinical applications in RA appears to be through the suppression of the proliferative capacity of T cells - although murine cells appear to require higher doses to achieve a significant effect.

We then assessed if tofacitinib's ability to inhibit cytokine signalling in $CD4^+$ T cells could interfere in the differentiation of T cells. Tofacitinib during priming *in vitro* or *in vivo* inhibited Th1 polarisation, evidenced by decreased T-bet expression – an effect that persisted upon a secondary antigen challenge – and/or IFN-γ production. In addition, tofacitinib treatment *in vitro* reduced the secretion of IL-5 by $CD4^+$ T cells, a Th2-specific cytokine. These findings come in line with the requirement of functional IFN-γ signalling to induce T-bet expression via activation of STAT1, which leads to enhanced IFN-γ production (23). As such, tofacitinib appears to disrupt the IFN-γ self-enhancing loop. Similar changes were detected following naïve murine $CD4^+$ T cell exposure to the drug, which exhibited a dose-dependent T-bet reduction comparable with the one seen in $CD4^+$ T cells from $STAT1^{-/-}$ mice (1). This study also demonstrates that tofacitinib inhibits IL-4-dependent Th2 differentiation, in addition to interfering with Th17 differentiation – although no differences in IL-17 were found in our assays. Additionally, the impact of tofacitinib in IL-5 secretion is believed to be mediated by inhibition of STAT3, as this transcription factor is required for Th2 development (24).

Piscianz *et al*. observed the recovered responsiveness of T cells to re-activation *in vitro* after tofacitinib withdrawal, and while some cytokine production remained inhibited, the production of IL-2, IL-13 and TNF-α was preferentially restored (17). Another study demonstrated that IFN-γ-producing cells collected from healthy individuals 4 weeks after termination of tofacitinib therapy recovered their responses to antigenic stimulation after transient function suppression during treatment (25). Moreover, $CD4^+$ T cells isolated from peripheral blood and synovium of patients with active RA exhibited a reduced capacity to produce IFN-γ upon CD3/CD28 stimulation in the presence of the drug *in vitro* (21). However, cells from these studies comprised of an heterogenous population of T cells, while the homogeneity of T cell populations used in our experiments allows a more precise evaluation of the drug's impact in the different stages of T cell activation. In our assays, tofacitinib did not impact T-bet expression or IFN-γ production by cells that were primed in the absence of drug and re-challenged in the presence of the drug. In addition to the previously demonstrated inhibition effects of tofacitinib during priming, these findings suggest that tofacitinib's mode of action in RA could not be mediated by manipulation of antigen experienced CD4 T cell outcomes, but possibly by an effect in recently primed CD4 T cell responses. We then analysed the drug's effects in the development of clinical disease and in recently primed and memory CD4 T cell responses in the context of early autoimmune arthritis.

Tofacitinib promoted a faster resolution of arthritis in our experimental murine model of autoimmune arthritis. This finding supports previous reports in the literature that utilised other early (2) or late (1, 3, 4) animal models of arthritis, in which tofacitinib's efficacy was attributed to an inhibition effect in the production of inflammatory mediators. When analysing the drug's impact on adaptive immune components that play a role in RA, such as responses by antigen experienced $CD4^+$ T cells, we found a decrease in the accumulation of exogenous T cells in the LNs. This effect is unlikely to be related to cell proliferation, as supported by our *in vitro* and *in vivo* data, but could be related to an impact in apoptosis or migration of effector T cells to the inflamed joint. Although we found a reduction in the secretion of a subclass of antibodies commonly produced during Th1 responses, our data does not support an interference of tofacitinib in Th1 activity, as the drug did not affect T-bet expression by these antigen experienced cells. As such, tofacitinib could have been impacting B cell activation (15, 16) or differentiation via its suppression effects on T follicular helper (Tfh) cells (26). On the other hand, we demonstrate that tofacitinib's capacity to modify T cell outcomes following $CD4^+$ T cell-DC interactions during priming limits breach of self-tolerance in autoimmune arthritis. The treatment inhibited the development of Th1 phenotype in recently primed endogenous T cells, some of which may have recognised auto-antigen (9). No effects were found in the proportion of memory T cells. The specificity of these memory cells is unknown and might be unrelated to the arthritic response (9, 27). On the other hand, if they are involved in this response, they might be more terminally differentiated and therefore take longer to be affected by tofacitinib, or even be refractory to the drug. The reduction in Th1 polarisation – possibly in auto-reactive T cells – may have led to the diminished anti-type II collagen responses found in our experimental model, which may have ultimately led to amelioration of clinical disease. As we have previously demonstrated, autoimmune components play a more significant role in a second footpad challenge when the current model is extended (27). As such, further studies utilising this "later" model of arthritis comparing clinical effects when the drug is introduced in different stages of disease development (*e.g*. induction phase *vs*. shortly before a second challenge) will further our understanding in the drug's effects in autoimmunity.

Taken together, our data provide mechanistic rationale informing how tofacitinib suppresses $CD4^+$ T cell immune responses, in the context of experimental autoimmune arthritis. In turn, these findings provide insights for future research directions on the early application of tofacitinib in RA.

Key messages

- The Janus kinase inhibitor tofacitinib inhibits Th1 polarisation during T cell priming but not re-activation.
- The disruption in T cell priming by tofacitinib limits breach of self-tolerance in experimental arthritis.

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